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[COMMUNICATION]

The Effect of Culture Plate Ventilation Space on Cell Growth *in vitro*

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ABSTRACT—Human fetal lung fibroblast cells (TIG-3) and HeLa cells were cultured in a multiplate either covered with a lid (lid^+) or, alternatively without a lid (lid^-). In case of TIG-3, proliferation of the cells in the lid^- plate was significantly less as compared with that in the lid^+ plate, regardless of the serum concentration. On the contrary, HeLa cells significantly increased in cell number in the lid^- plate. Two kinds of culture media, one supplemented with fetal bovine serum and the other supplemented with the freeze-dried fetal bovine serum, were compared regarding the effect of the lid. The type of culture medium did not affect the results. Production of CO_2 during the culture period did not affect the CO_2 concentration of the atmosphere of the culture flask, suggesting that the phenomenon was not due to the CO_2 concentrations. The explanation of the result may be as follows: A highly volatile growth regulating factor(s) was secreted from the cells, acting as either promotive or inhibitory depending upon the responsiveness of the cell types.

INTRODUCTION

Although many cell-growth related substances have been discovered, in the animal (especially normal) cell culture system, any number of combinations of those can not so far promote a universally satisfactory cell-growth equivalent to that with sera. In addition, even in a satisfactory medium supplemented with serum, normal cells generally fail to grow or even die when the cell density is very low. Those facts imply that sera probably contain more unknown growth related factors and that cell themselves may secrete one or more autostimulating factors not derived from sera. When human dermal fibroblast cells (HDF: freshly prepared from a healthy adult man) were inoculated in 96 wells multiplate at a low cell density and with a low concentration of fetal bovine serum, we noticed that the cells in the corner of the plate proliferated less than those in the center. We thus carried out an experiment sealing the margin of the plate after the equilibration of the gas phase, resulting in a demonstration of homogeneous growth throughout all the holes of the multiplate [1]. This phenomenon may be explained as follows: A volatile growth-promoting factor having been secreted by an autocrine or similar mechanism, might easily evaporate at 37°C and gradually diffuse through the plate space where the atmospheric concentration of the factor was presumably higher at the center but lower at the corner. In the present experiment, we used two groups of plates, one with lids and the other without them, and the proliferation rates of the cells were compared.

MATERIALS AND METHODS

Both human fetal lung fibroblasts (TIG-3) and HeLa cells (both supplied from the JCRB Cell Bank) had been passaged in the Eagle's MEM supplemented with 10% fetal bovine serum (FBS) before use. TIG-3 cells presently used were at 20 to 40 passages (split ratio:1/2). The cells were inoculated in 24 well multiplates (1000 μl medium/well) at a population of 10,000 cells/well and

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cultured for four days in a humidified CO_2 incubator regulated at 37°C, 5% CO_2 and 95% air. The media used were Eagle's MEM supplemented with 5–20% FBS or freeze-dried FBS. When the lid was removed, evapolation loss of the water from the media slightly but significantly occurred after four days of culture despite of humidification of the incubator. Water was therefore daily added to the media (20–25 μl/1000 μl) to compensate for the loss. Each of the osmotic pressure (using an osmometer 3w2: Advanced Instlement Corp.), volume of the media and the pH were measured after four days of culture in all the experiments to ascertain the uniformity of water and hydrogen ion contents among the media. The CO_2 concentration of the gas phase in the flask was measured with an infrared CO_2 analyzer (Fuji Electric Co. model Z-AU) by infusion of gas with a gas syringe into a constant air flow. A Coulter counter was used for counting the number of cells. Results were expressed as mean $\pm$ SE for each set of 8 data points, and statistical significance was culculated using Studen's *t*-test.

RESULTS

Effect of the lid on the cell proliferation

Figure 1 shows the growth of TIG-3 and HeLa cells with serum not freeze-dried after four days of culture. TIG-3 significantly decreased in the final cell number when the lid was removed, regardless of the serum concentration. On the contrary, HeLa cells significantly increased in cell number in the same lid$^-$ condition, and reached the same level independently of the serum concentration. Almost the identical result was obtained in the experiment with freeze-dried serum (data not shown).

CO_2 concentration of the gas phase in the culture flask during culture

Culture flasks (25 cm^2) with 40,000 or 320,000 of TIG-3 or HeLa cells were equilibrated with 5% CO_2 and 95% air and then capped tightly. The cells thus confined were cultured for five days during which CO_2 concentration of the gas phase in the flask was monitored with an infrared spectrometer. No significant increase in CO_2 concentration was observed during the five days of culture, suggesting that the lid effect was not due to the retention of the respiratory CO_2.

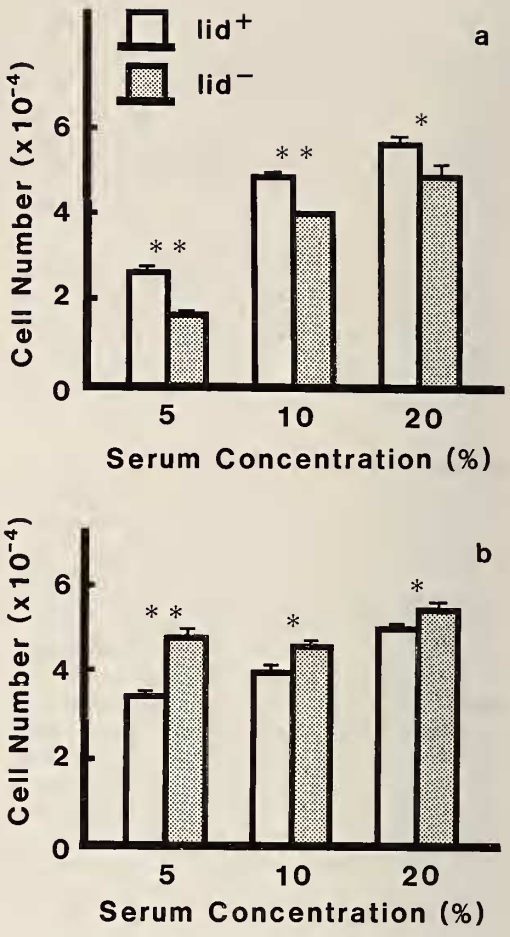

FIG. 1. Growth of TIG-3 cells (a) and HeLa cells (b) either covered with lid (open bars) or without lid (dotted bars) in MEM supplemented with 5–20% FBS (not freeze-dried) at a cell number of 10,000. After four days of culture, cells were trypsinized and the numbers were counted by a Coulter counter. *: Differences of growth between lid$^+$ vs lid$^-$ were significant as **: $p<0.001$, *: $p<0.01$.

DISCUSSION

The present experiment demonstrated that the cover lid of the culture plate has an important role for cell proliferation besides prevention of bacterial contamination. Physical conditions for the cell

culture such as pH, CO_2 concentration or osmotic pressure have long been discussed and reviewed by a number of researchers [2]. Each TIG-3 and HeLa cells presently used showed no significant difference in cell proliferation rate under the following range of conditions of the pH (7.0–7.4), the CO_2 concentration (3–10%) and the osmotic pressure (270–300 mosm/kgH_2O) tested as a preliminary experiment. The pH, the CO_2 concentration and the osmotic pressure monitored after each of the experiments as described in the materials and methods section showed constant values of 7.2, 5% and 280 mosm/kgH_2O respectively. Accordingly no such conditions seemed to affect the present results. The mechanism of the lid effect is yet unknown; however, the present data suggest that the cover lid may prevent escape of a volatile factor related to cell proliferatio. This factor, if it exists, may have both promoting and inhibiting effects on cell growth depending upon cell types, presumably normal cells and cancer cells respectively. Another possibility is that the factor may have a concentration-related biphasal effect, either promotive or inhibitory, according to the responsiveness of the cell types. TIG-3 may secrete a different factor from that of the HeLa cells. The factor is probably not contained in the serum but produced by the cells themselves since the same phenomenon occurs even when freeze-dried FBS is used. If the factor is autocrined from cells, the present result may explain why normal cells generally fail to grow or even die under a condition of very low population, and also why they are very difficult to clone unless a feeder layer [3] is used. The physiological aspects of the present results are to be considered along with the background information that cells of multicellular organisms are generally placed in an enclosed environment, and the concentration of the soluble materials in which cells are located should be properly balanced by their supply and removal through degradation, exclusion and expiration. Purification of the factor is now being attemped by collecting crude samples with a liquid nitrogen trap and further separation with gas chromatography.

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